Synthesis of a Series of Stromelysin-Selective Thiadiazole Urea Matrix Metalloproteinase Inhibitors

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The synthesis and enzyme inhibition data for a series of thiadiazole urea matrix metalloproteinase (MMP) inhibitors are described. A broad screening effort was utilized to identify several thiadiazoles which were weak inhibitors of stromelysin. Optimization of the thiadiazole leads to include an α-amino acid side chain with variable terminal amide substituents provided a series of ureas which were moderately effective stromelysin inhibitors, with K_i's between 0.3 and 1.0 μM. The most effective analogues utilized an L-phenylalanine as the amino acid component. In particular, unsubstituted **46** had a K_i of 710 nM, while the *p*-fluoro analogue **52** displayed increased potency (100 nM). Stromelysin inhibition was further improved using a pentafluorophenylalanine substituent which resulted in **70**, a 14 nM inhibitor. While gelatinase inhibition was generally poor, the use of 1-(2-pyridyl)piperazine as the amide component usually provided for enhanced activity, with **71** inhibiting gelatinase with a K_i of 770 nM. The combination of this heterocycle with a *p*-fluorophenylalanine substituent provided the only analogue, **69**, with collagenase activity (13 μM). The SAR for analogues described within this series can be rationalized through consideration of the X-ray structure recently attained for **70** complexed to stromelysin. Uniquely, this structure showed the inhibitor to be completely orientated on the left side of the enzyme cleft. These results suggest that thiadiazole urea heterocycles which incorporate a substituted phenylalanine can provide selective inhibitors of stromelysin. Careful selection of the amide substituent can also provide for analogues with modest gelatinase inhibition.

Introduction

The matrix metalloproteinases (MMPs) are a family of zinc endopeptidases which are capable of degrading the extracellular matrix of connective tissues and basement membranes.[1] These proteinases are involved in tissue remodeling and are generally regulated by selective endogenous tissue inhibitors of metalloproteinases (TIMPs).[2] Overactivation or increased synthesis of the MMPs has been implicated in several disease pathologies.[3] There are currently at least 18 members of this family of proteinases which can be roughly associated into three groups depending on their native substrates. Thus, the gelatinases are effective proteinases of type IV collagen, the collagenases degrade interstitial collagen, and the stromelysins are effective as protoglycanases. The discovery and characterization of the MMPs are summarized in several recent reports.[4]

MMPs have been implicated in a number of disease states, including arthritis, cancer progression, and related connective tissue disorders. For instance, increased levels of MMPs (collagenase, stromelysin) have been noted in cartilage and synovial fluid in arthritis with an excellent correlation of the levels of the MMPs to severity of disease.[5] Recent studies with cancer patients have documented that overexpression of MMPs is directly correlated with life span.[6,7] Other possible therapeutic implications for MMP inhibitors (MMPIs) include periodontal disease,[8] atherosclerotic plaque rupturing,[9] and aortic aneurysms.[10] Recently several research groups have shown that related metalloproteinases are also involved in the conversion of inactive tumor necrosis factor-α (TNF-α) into active TNF.[11,12] Usually this inflammatory cytokine plays a beneficial role in physiological defense responses; however, overproduction of TNF-α can lead to systemic toxicity. Therefore, MMP inhibitors may be indirectly involved in diseases for which TNF-α has been implicated, such as Crohn's disease, MS, cachexia, sepsis, and rheumatoid arthritis.[13]

Recent efforts by a number of laboratories have provided several classes of MMP inhibitors which have been extensively reviewed.[14−16] In general, each class contains a zinc ligand attached to a small peptide fragment which is capable of binding to specificity pockets of the MMP enzymes, usually on the P′ side. The zinc ligands utilized most frequently are hydroxamates, thiols, carboxylic acids, and phosphoric acids. In general, the use of hydroxamates as the zinc ligand provides for the most effective MMP inhibitors. However, hydroxamates are often found to be biologically labile which has prompted additional efforts toward the discovery of new chelating groups suitable for use in

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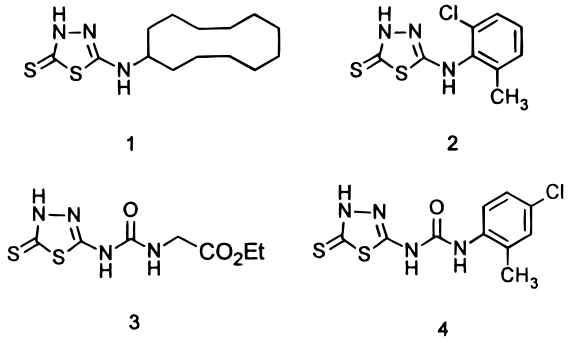


Figure 1. Thiadiazole screening leads.

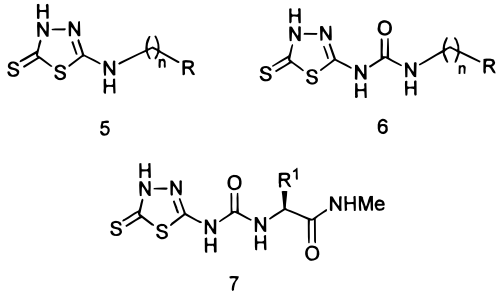
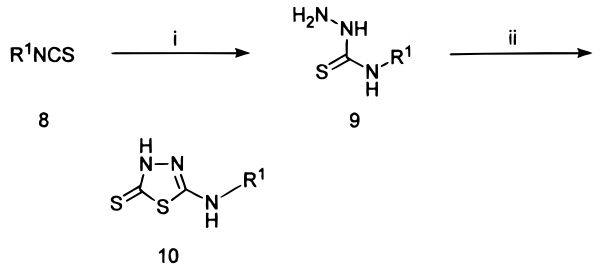


Figure 2. Thiadiazole amine and urea targets.

MMPI templates.[17] To explore this issue further, a broad screening effort was undertaken using stromelysin as the screening enzyme. From this, a number of chemical classes were identified which were weak (>50 μM) inhibitors of stromelysin. Of these, several members of the thiadiazole class (Figure 1, amines and ureas) were determined to be competitive stromelysin inhibitors.

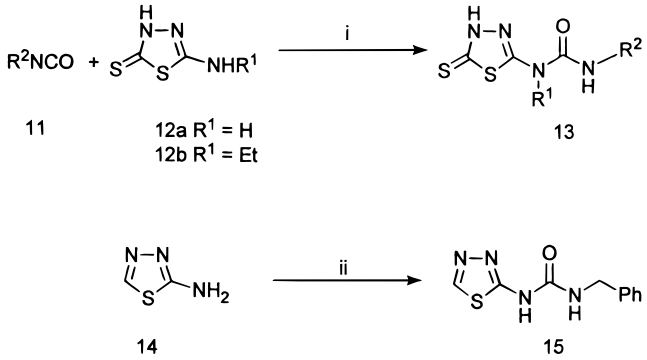
In this report we describe the elaboration of this series to improve enzyme potency, evaluate structure−activity relationship (SAR), and determine MMP specificity. In particular, we were interested in the exploration of compounds in the amine (**1**, **2**) and urea (**3**, **4**) classes. During this SAR elaboration of the thiadiazole screening leads, other reports of thiadiazole-based MMP inhibitors have appeared.[18] Additional effort toward the development of an amide thiadiazole series is reported separately.[19] Under the assumption that the exo-cyclic sulfur chelates to the catalytic zinc, with the side chain of **1**−**4** generally located toward the S′ side, several analogues were targeted. By application of known peptidic SAR,[14−16] several compounds containing groups capable of interacting with the S2′−S3′ subsites of stromelysin were designed (see Figure 2). While it was desirable to also incorporate a P1′ group, the steric bulk of the thiadiazole heterocycle and linking group (amine, urea) appeared to effectively prevent this. In addition, exploration of the thiadiazole core unit was initially of interest. However, as related non-thiadiazole heterocycles were ineffective stromelysin inhibitors (also see Discussion section), this effort was abandoned. Because of the lack of similarity between the thiadiazoles and known peptide mimetics,[14−16] it was extremely difficult to have confidence in these predictions, despite the appearance of stromelysin/inhibitor complexes[20,21] and modeling studies, as these leads were so novel. Overall, through a "loose" (albeit incorrect, vide infra) application of peptidic hydroxamate SAR, this effort led to a series of thiadiazole benzenepropanamides, of which the best

Scheme 1. Synthesis of Thiadiazole Amines[a]



[a] Reagents: (i) H_2NNH_2, EtOH; (ii) CS_2, DMF.

Scheme 2. Synthesis of Simple Thiadiazoles[a]



[a] Reagents: (i) THF; (ii) $PhCH_2NCO$, THF.

compounds represented over a 4700-fold improvement in stromelysin inhibition, as compared to the screening leads.

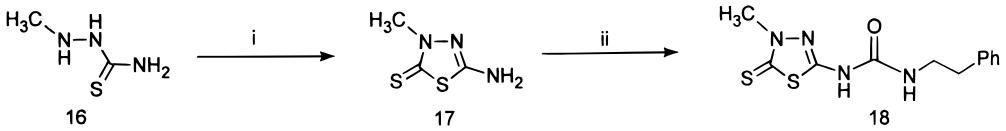
Chemistry

The general synthesis of the thiadiazole amines **10** following standard procedures[22] is illustrated in Scheme 1. Reaction of the desired isothiocyanate **8** with hydrazine hydrate provided thiosemicarbazide **9** which was treated directly with carbon disulfide in DMF at 60 °C to give aminothiadiazole **10**.

The thiadiazole urea analogues were synthesized as shown in Schemes 2−5. The simple thiadiazole ureas **13** were generally prepared by condensation of **11** with 2-amino-1,3,4-thiadiazole-2-thiol (**12a**) as shown in Scheme 2. In one case, an N-substituted thiadiazole (**12b**) was used. The isocyanates **11** utilized were either commercially available or were generated by Curtius rearrangement[23] of the corresponding carboxylic acid and used in situ. A desthiocarbonyl analogue (**15**) was also prepared within this series. Treatment of **14** with benzyl isocyanate provided **15**. To further evaluate the role that the thiol tautomer may play in MMP inhibition, a 3-methyl analogue (**18**) was prepared as shown in Scheme 3. Reaction of 1-methylthiosemicarbazide **16**, prepared by Jensen's procedure,[24] with thiocarbonyldiimidazole in warm DMF gave **17**. Exposure of this heterocycle to phenethyl isocyanate, as described above for **12a**, did not provide any of the desired product. However, treatment of **17** with sodium hydride followed by phenethyl isocyanate afforded **18** in good yield.

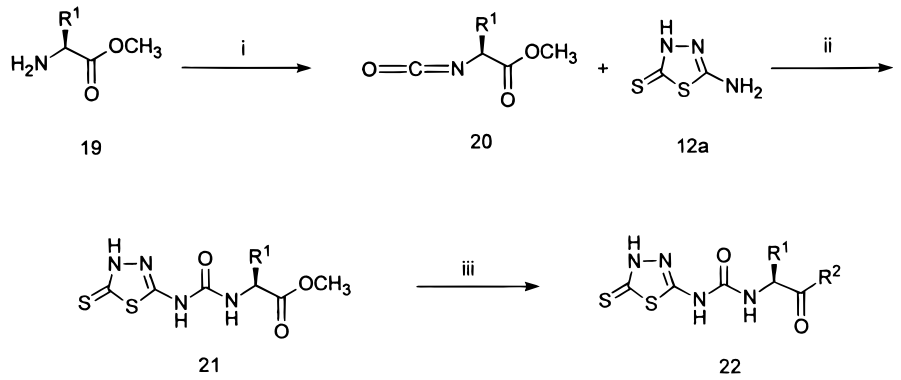
The general strategy for the preparation of **13** was readily adapted toward the synthesis of the more complex, amino acid-derived thiadiazoles as depicted in Scheme 4. The isocyanate of the desired amino acid **20** was formed by treating amino acid methyl ester **19** with phosgene and pyridine, following the procedure recently

Scheme 3. Synthesis of Urea **18**[a]



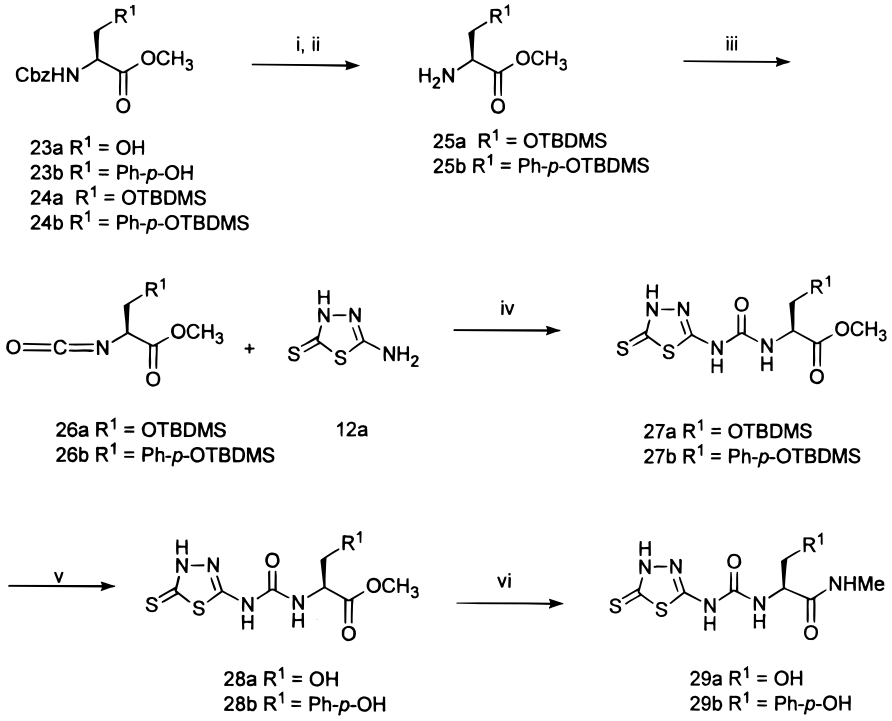
[a] Reagents: (i) 1,1′-thiocarbonyldiimidazole, THF; (ii) NaH, THF, $PhCH_2CH_2NCO$.

Scheme 4. Synthesis of Amino Acid-Derived Thiadiazole Ureas[a]



[a] Reagents: (i) ClCOCl, CH_2Cl_2, pyridine; (ii) THF; (iii) R_2NH_2, EtOH.

Scheme 5. Synthesis of Amino Acid-Derived Thiadiazoles[a]

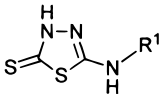


[a] Reagents: (i) *t*-BDMSCl, imidazole, DMF; (ii) ammonium formate, Pd/C, MeOH; (iii) ClCOCl, CH_2Cl_2, pyridine; (iv) THF; (v) Bu_4NF, THF; (vi) 40% $MeNH_2$.

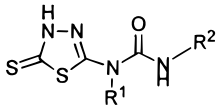
outlined by Nowick.[25] The prerequisite methyl ester starting materials **19** were either commercially available or prepared by standard methods. The reaction of isocyanate **20** with thiadiazole **12a** in THF provided urea **21** in acceptable yield. The ester analogues were converted to amide **22** by treatment of **21** with the desired amine, either neat or diluted with ethanol. Nowick reported[25] that no racemization occurred during the formation of **20**. However, some loss of chirality is possible during the conversion of the isocyanate to **21**, as well as the formation of amide **22** from ester **21**. Chiral HPLC analysis (Welk-O (R,R) column; 30% 2-propanol/hexane) of **46** and *rac*-**46** indicated that no racemization had occurred. Other analogues were not evaluated.

Analogues containing either a serine or tyrosine amino acid derived-thiadiazole were synthesized as shown in Scheme 5. Protection of N-CBZ-L-serine methyl ester (**23a**) or N-CBZ-L-tyrosine methyl ester (**23b**) with *tert*-butyldimethylsilyl chloride provided silyl ethers **24a** and **24b**. Deprotection of these carbamates under transfer hydrogenation conditions provided amines **25a** and **25b**. Conversion of these amines to isocyanates **26a** and **26b**, as detailed above, using phosgene and pyri-

Table 1. Physical Data for Thiadiazole Amines and Ureas



amine-10

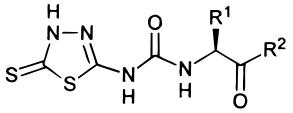


urea-13

compd	core structure	R^1	R^2	mp (°C)	formula	anal.
12b	**10**	Et		139−140	$C_4H_7N_3S_2$	C, H, N
31	**10**	Ph		213−215	$C_8H_7N_3S_2$	C, H, N
32	**10**	CH_2Ph		138−141	$C_9H_9N_3S_2$	C, H, N
33	**10**	$(CH_2)_2Ph$		146−148	$C_{10}H_{11}N_3S_2$	C, H, N
34	**10**	$(CH_2)_4Ph$		117−119	$C_{12}H_{15}N_3S_2$	C, H, N
35	**10**	CH_2-2-furyl		131−133	$C_7H_7N_3S_2$	C, H, N
36	**13**	H	Ph	227−228	$C_9H_8N_4S_2O$	C, H, N
37	**13**	H	4-MeO-Ph	235−237	$C_{10}H_{10}N_4S_2O_2$	C, H, N
38	**13**	H	4-F-Ph	232−233	$C_9H_7N_4S_2OF$	C, H, N
39	**13**	H	CH_2Ph	200−202	$C_{10}H_{10}N_4S_2O$	C, H, N
15[a]	**13**	H	CH_2Ph	235−237	$C_{10}H_{10}N_4SO$	C, H, N
40	**13**	H	$(CH_2)_2Ph$	196−198	$C_{11}H_{12}N_4S_2O$	C, H, N
18[b]	**13**	H	$(CH_2)_2Ph$	222−223	$C_{12}H_{14}N_4S_2O$	C, H, N
41	**13**	Et	$(CH_2)_2Ph$	153−154	$C_{13}H_{16}N_4OS_2$	C, H, N
42	**13**	H	$(CH_2)_3Ph$	176−178	$C_{12}H_{14}N_4S_2O$	C, H, N
43	**13**	H	$(CH_2)_4Ph$	175−177	$C_{13}H_{16}N_4S_2O$	C, H, N
44	**13**	H	CH_2-naphth	198−201	$C_{14}H_{12}N_4S_2O$	C, H, N

[a] See Scheme 2. [b] See Scheme 3.

Table 2. Physical Data for Thiadiazole Ureas



22

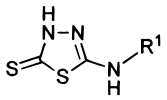
compd	R^1	R^2	mp (°C)	method	yield (%)	formula	anal.
45	H	NHMe	244−246	B	63	$C_6H_9N_5O_2S_2\cdot(H_2O)_{1/3}$	C, H, N
46	CH_2Ph	NHMe	234−236	B	51	$C_{13}H_{15}N_5O_2S_2\cdot(H_2O)_{1/4}$	C, H, N
***ent*-46**	*ent*-CH_2Ph	NHMe	228	B	81	$C_{13}H_{15}N_5O_2S_2$	C, H, N
47	CH_2cyclohexane	NHMe	246−247	B	77	$C_{13}H_{21}N_5O_2S_2\cdot(CH_2Cl_2)_{1/8}$	C, H, N
48	*i*Bu	NHMe	110−112	B	41	$C_{10}H_{17}N_5O_2S_2\cdot(H_2O)_{1/4}$	C, H, N
49	Ph	NHMe	240−241	B	23	$C_{12}H_{13}N_5O_2S_2$	C, H, N
29a	CH_2OH	NHMe	192	B	49	$C_7H_{11}N_5O_3S_2\cdot(MeOH)_{1/2}$	C, H, N
50	CH_2OCH_2Ph	NHMe	195−196	B	47	$C_{14}H_{17}N_5O_3S_2\cdot(H_2O)_{1/3}$	C, H, N
51	CH_2Ph	OMe	102−105	A	58	$C_{13}H_{14}N_4O_3S_2\cdot(C_6H_{14})_{1/8}$	C, H, N
52	CH_2Ph-*p*-F	NHMe	225−227	B	54	$C_{13}H_{14}FN_5O_2S_2\cdot(C_4H_8O_2)_{1/8}$	C, H, N
29b	CH_2Ph-*p*-OH	NHMe	198−200	B	73	$C_{13}H_{15}N_5O_3S_2\cdot(H_2O)_{1/2}\cdot(C_4H_2O_2)_{1/2}$	C, H, N
53	CH_2Ph-*p*-OMe	NHMe	232−233	B	94	$C_{14}H_{17}N_5O_3S_2$	C, H, N
54	CH_2Ph-*p*-NO_2	NHMe	228−230	B	91	$C_{13}H_{14}N_6O_4S_2$	C, H, N
55a	CH_2Ph	OH	114−116		97	$C_{12}H_{12}N_4O_3S_2\cdot(H_2O)_{1/4}$	C, H, N
56	CH_2Ph	NH_2	175−177	B	31	$C_{12}H_{13}N_5O_2S_2\cdot(CH_4O)_{1/4}$	C, H, N
57	CH_2Ph	NMe_2	196−197	B	61	$C_{14}H_{17}N_5O_2S_2$	C, H, N
58	CH_2Ph	NH*i*Pr	206−211	C	77	$C_{15}H_{19}N_5O_2S_2$	C, H, N
59	CH_2Ph	$NHCH_2Ph$	198−200	C	61	$C_{19}H_{19}N_5O_2S_2\cdot(H_2O)_{1/8}$	C, H, N
60	CH_2Ph	veratrylamine	207−210	B	34	$C_{21}H_{23}N_5O_4S_2$	C, H, N
61	CH_2Ph	$NHCH_2$cyclohexane	185	C	85	$C_{19}H_{25}N_5O_2S_2\cdot(H_2O)_{1/6}$	C, H, N
62	CH_2Ph	morpholine	213−216	C	79	$C_{16}H_{19}N_5O_3S_2\cdot(H_2O)_{2/5}$	C, H, N
63	CH_2Ph	pyrrolidine	185	C	63	$C_{16}H_{19}N_5O_2S_2\cdot(H_2O)_{1/3}$	C, H, N
64	CH_2Ph	thiomorpholine	210−214	C	50	$C_{16}H_{19}N_5O_2S_3$	C, H, N
65	CH_2Ph	*N*-benzylpiperazine	210−213	C	30	$C_{23}H_{26}N_6O_2S_2\cdot(H_2O)_{1/4}$	C, H, N
66	CH_2Ph	1-(2-pyridyl)piperazine	220−222	C	67	$C_{21}H_{23}N_7O_2S_2$	C, H, N
67	CH_2Ph	1-(2-pyrimidinyl)piperazine	235−237	B	56	$C_{20}H_{22}N_8O_2S_2$	C, H, N
68	CH_2Ph	4-MeO-phenylpiperazine	201−203	B	67	$C_{23}H_{26}N_6O_3S_2$	C, H, N
69	CH_2Ph-*p*-F	1-(2-pyridyl)piperazine	240−241.5	B	55	$C_{21}H_{22}FN_7O_2S_2\cdot(H_2O)_{1/8}$	C, H, N
70	$CH_2C_6F_5$	NHMe	229−232	B	39	$C_{13}H_{10}F_5N_5S_2O_2$	C, H, N
71	$CH_2C_6F_5$	1-(2-pyridyl)piperazine	233−235	B	46	$C_{21}H_{18}F_5N_7O_2S_2\cdot(H_2O)_{1/3}$	C, H, N

dine, proceeded uneventfully. Exposure of each to **12a** provided thiadiazoles **27a** and **27b**. Each of these intermediates was deprotected using tetrabutylammonium fluoride to provide the hydroxyl derivatives. The methylamide analogues **29a** and **29b** were prepared under the standard conditions. The physical data for all analogues synthesized are shown in Tables1 and 2.

Results and Discussion

Three slightly different assays were utilized for enzyme inhibition studies of the analogues reported herein. An enzymatic assay using β-casein as the substrate was utilized in the broad screening effort. This particular assay was also used for the evaluation of

Table 3. Stromelysin Inhibition for Thiadiazole Amines and Ureas



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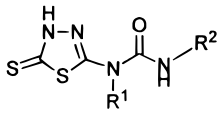
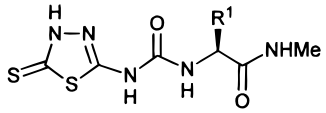


13

compd	core structure	R^1	R^2	IC_{50} str (μM)[a]	K_i str (μM)[b]
12b	**10**	Et		>2000	
31	**10**	Ph		190	
32	**10**	CH_2Ph		97	
33	**10**	$(CH_2)_2Ph$		47	
34	**10**	$(CH_2)_4Ph$		318	
35	**10**	CH_2-2-furyl		289	
36	**13**	H	Ph	99	
37	**13**	H	4-MeO-Ph		69
38	**13**	H	4-F-Ph	29	
39	**13**	H	CH_2Ph		16
44	**13**	H	CH_2-naphth	23	
15 (desthio)	**13**	H	CH_2Ph	>2000	
40	**13**	H	$(CH_2)_2Ph$		7
18 (4-methyl)	**13**	H	$(CH_2)_2Ph$	694	
41	**13**	Et	$(CH_2)_2Ph$	>1000	
42	**13**	H	$(CH_2)_3Ph$		16
43	**13**	H	$(CH_2)_4Ph$	34	
1	**10**	$C_{12}H_{23}$		97	
2	**10**	2-Cl-6-Me-Ph		173	
3	**13**	H	CH_2CO_2Et	143	85
4	**13**	H	4-Cl-2-Me-Ph	125	119

[a] These analogues were evaluated against stromelysin-1 using the screening assay. IC_{50} values are provided. [b] These compounds were evaluated against stromelysin-1 using the continuous fluorescence assay. K_i values are provided.

Table 4. Stromelysin and Gelatinase A Inhibition for Thiadiazole Urea Methylamides



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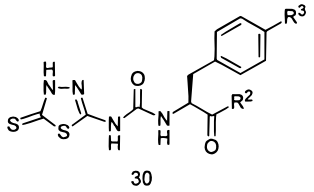
compd	R^1	K_i str (μM)[a]	K_i gel (μM)[a]
45	H	166	>100
46	CH_2Ph	0.71	31
***ent*-46**	*ent*-CH_2Ph	5.3	>200
47	CH_2cyclohexane	4.1	67
48	*i*Bu	17	>200
49	Ph	4.1	41
29a	CH_2OH	31	>200
50	CH_2OCH_2Ph	2.0	52
3		85	

[a] All analogues were evaluated against stromelysin-1 and gelatinase A using the PCA assay. K_i values provided.

Table 5. Stromelysin and Gelatinase A Inhibition for Thiadiazole Ureas



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compd	R^2	R^3	K_i str (μM)[a]	K_i gel (μM)[a]
46	NHMe	H	0.71	31
51	OMe	H	0.29	17
52	NHMe	F	0.10	8.9
29b	NHMe	OH	0.40	21
53	NHMe	OMe	0.46	16
54	NHMe	NO_2	0.33	15
55	OH	H	3.2	>200
56	NH_2	H	3.3	>200
57	NMe_2	H	2.3	226
58	NH*i*Pr	H	1.3	
59	$NHCH_2Ph$	H	0.51	15
60	veratrylamine	H	0.49	6.2
61	$NHCH_2$cyclohexane	H	0.81	>200
62	morpholine	H	0.93	32
63	pyrrolidine	H	0.66	20
64	thiomorpholine	H	0.38	12
65	*N*-benzylpiperazine	H	0.86	29
66	(2-pyridyl)piperazine	H	0.52	1.5
67	2-(pyrimidinyl)piperazine	H	0.42	16
68	4-MeO-phenylpiperazine	H	1.0	6.0
69	(2-pyridyl)piperazine	F	0.27	1.8
70	NHMe	pentafluoro[b]	0.018	3.0
71	(2-pyridyl)piperazine	pentafluoro[b]	0.014	0.77

[a] All analogues were evaluated against stromelysin-1 and gelatinase A using the PCA assay. K_i values are provided. [b] Ph-$R_3 = C_6F_5$.

many of the thiadiazole analogues reported in Table 3. A number of the thiadiazoles listed in Table 3 were evaluated against stromelysin using a continuous fluorescence inhibition assay. In addition, a particle concentration fluorescent assay (PCFA) was developed based on that reported by Manndetta[26] for screening HIV protease inhibitors. This assay was used for the evaluation of the analogues reported in Tables 4 and 5. Most analogues were also evaluated for their ability to inhibit TNF-α production after an endotoxin challenge.[27]

Our initial screening efforts focused on the thiadiazole amines (**10**) and thiadiazole ureas (**13**). As shown in Table 3, little improvement in activity was observed for the amine analogues as compared to **1** and **2**. For example in comparison to **2**, only a 4-fold increase in MMP inhibition was attained with **33**, a 47 μM inhibitor. Substituents on the aryl ring of **2** were not critical, as **31** was equipotent. Only the benzyl (**32**) and phenethyl (**33**) analogues represented a modest improvement in activity over **2**. The *N*-ethyl analogue, **12b**, was

completely inactive. The inhibition of stromelysin was slightly improved for compounds in the urea series (**13**). By simply varying the chain length and substitution pattern of **4**, roughly a 17-fold improvement in stromelysin inhibition was noted. In this case, **40**, with a 2-carbon side chain, was the most effective inhibitor, with a K_i of 7 μM. As compared to **4**, both the benzyl (**39**) and phenpropyl (**42**) analogues also displayed increased potency. Extending the side chain further resulted in diminished potency, although bulky substituents (**44**) were still tolerated. Like **4**, phenyl **36** and the 4-methoxy analogue **37** were weak inhibitors. Interestingly, incorporation of a 4-fluoro group (**38**) did result in a slight (ca. 3-fold) improvement in stromelysin inhibition over **4** and **36**. The thiocarbonyl group of **40** is essential, as the desthio isomer **15** was devoid of activity. Additionally, methyl substitution at the aryl urea nitrogen (**41**), or the 3-nitrogen on the thiadiazole ring (**18**), led to a complete loss of activity. These results suggest that these hydrogens are necessary for enzyme interaction. It is also possible that the 100-fold loss in activity of **18** as compared to **40** is due to the requirement of the thio tautomer.

One of our primary interests in the thiadiazole urea series was to develop a SAR around **3**, the most potent of the screening leads. In particular, we were interested in the incorporation of a L-amino acid side chain, effectively providing **7**. Compounds of this nature were roughly designed[29] through consideration of the MMP peptide literature[14−16] and modeling efforts, which indicated that the R^1 and R^2 groups of **7** could be accommodated by the S2′ and S3′ pockets, respectively. Shown in Table 4 is a quick survey of thiadiazole amides derived from a series of L-amino acids. While the simple methylamide (**45**) was relatively inactive (K_i 166 μM) like **3**, introduction of a phenylmethyl R^1 ("phe") group provided a significant improvement in activity, with **46** having a K_i of 0.71 μM. The corresponding enantiomer, *ent*-**46**, was less potent, although with a K_i of 5.0 μM was not only surprisingly more effective than the screening leads but also equipotent to **40**. Analogues derived from several other amino acids were at least 3-fold less active, with **48** and serine **29a** particularly ineffective. Several compounds within this series did display minimal activity against gelatinase, with **46** having a K_i of 31 μM. None of these analogues were effective collagenase inhibitors.

With the moderate increase in potency for **46** over the screening leads, a number of analogues were evaluated which had different substituents at the methylamide position (R^2, see **30**). In addition, several functional groups at the *para*-position of the aryl ring (R^3) were explored, as shown in Table 5. Both the primary amide (**56**) and dimethylamide (**57**) derivatives were ineffective as compared to **46**, with K_i's of 3.3 and 2.3 μM, respectively. Surprisingly, the methyl ester **51** was an effective MMPI,[28] more potent than **46**. In comparison to **40**, incorporation of an ester group resulted in a 23-fold enhancement in stromelysin inhibitory activity. The corresponding carboxylic acid **55** was also slightly more active than **40**. The potency of **51** as compared to **46** is contradictory to the MMP peptide literature, where a secondary amide is generally required at P3′, since both the NH and carbonyl group of the amide participate in hydrogen-bonding interactions.

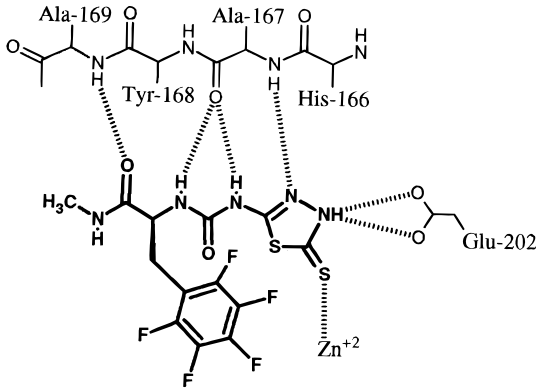

Figure 3. Hydrogen bonding between thiadiazole inhibitor **70** and the stromelysin active site. Dashed lines indicate potential hydrogen bonds between protein and inhibitor heteroatoms. Hydrogen positions must be inferred from heavier atom positions.

A number of other secondary and tertiary amides were evaluated within this series. In general, a relatively flat SAR was observed with the secondary amides inhibiting stromelysin 0.5−1.3 μM. The tertiary amines were slightly more effective, having K_i's of 0.39−1.0 μM. Interestingly, several compounds were identified which had moderate activity against gelatinase. Of these, **60**, **66**, and **68** were the most effective (1.5−6.0 μM). Thiadiazole **66**, which contained a 1-(2-pyridyl)piperazine amide, was unique, inhibiting stromelysin at 0.50 μM and gelatinase at 1.5 μM. Several analogues which had *para*-substituents on the aryl ring, while maintaining a methylamide (R^2 = NHMe), were also evaluated. Of these, the *p*-fluoro analogue **52** differentiated itself from the rest, inhibiting stromelysin at 100 nM and gelatinase at 8.9 μM. Compounds with other *para*-substituents displayed activity similar to **46**. Combining a *p*-fluoro R^3 group with a 1-(2-pyridyl)piperazine R^2 substituent provided **69**, which was the only compound from this series to have modest collagenase inhibitory activity (13 μM). To follow up on fluoro **69**, and to support NMR structure elucidation efforts,[29] two pentafluorophenyl analogues incorporating either a methyl or a 1-(2-pyridyl)piperazine amide substituent were prepared and evaluated. Both of these compounds were extremely effective stromelysin inhibitors (<20 nM). In addition, 1-(2-pyridyl)piperazine **71** inhibited gelatinase with a K_i of 770 nM, while methyl **70** was less effective, at 3.0 μM. Overall, while the terminal amide substituent could be widely varied, only the 1-(2-pyridinyl)piperazine group provided unique activity against gelatinase. In addition, a pentafluoro-substituted aromatic ring (**70**) was required for exceptional potency against stromelysin. None of the thiadiazole analogues that were tested inhibited the proteolytic conversion of the 26-kDa TNF-α precursor to the 17-kDa soluble cytokine.

When this work was nearly complete, an X-ray structure of **70** complexed to stromelysin[30] was elucidated. Much to our surprise, as shown in Figures 3 and 4, **70** bound to stromelysin completely on the unprimed side of the enzyme substrate binding cleft. As depicted in Figure 3, the thiocarbonyl interacts with the catalytic zinc with the remainder of **70** located toward the S1−S3 portion of stromelysin. The NH on the thiadiazole

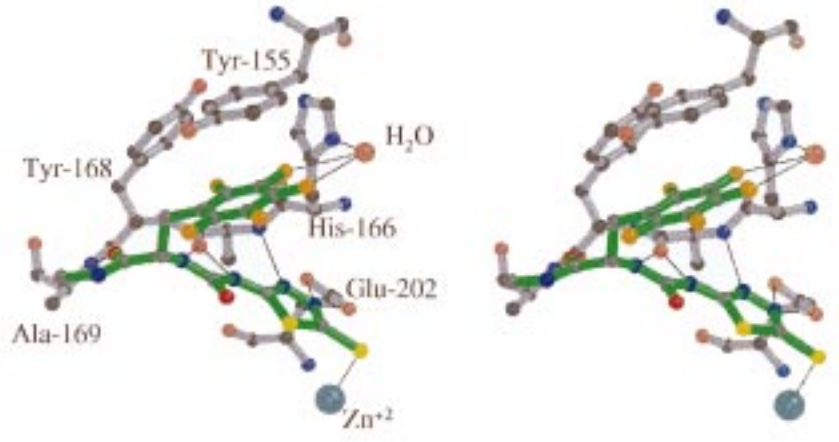

Figure 4. View of **70** bound to stromelysin. Hydrophobic interactions in the P1 and P3 enzyme binding sites are illustrated.

core participates in a bifurcated hydrogen bond to the carboxylate of glutamate 202. The other thiadiazole nitrogen (N_4) accepts a hydrogen bond from alanine 167. Furthermore, both urea hydrogens participate in a hydrogen bond to the carbonyl of alanine 167 on the stromelysin backbone. The unique hydrogen bonding of the thiadiazole core, in part, indicates why **15**, **18**, and **41** were ineffective stromelysin inhibitors, as well as why other related heterocycles were not identified through the screening effort. Furthermore, the general requirement of a phenethyl side chain (i.e., **40**, **46**) is obvious from Figure 4, where this group participates in a key $\pi-\pi$-stacking interaction with tyrosine 155. Fluoro substitution on the phenyl can enhance this interaction and also provide a hydrogen bond to the structural water located near histidine 166. Presumably both features contribute to the exceptional potency of the pentafluoro analogues (**70**, **71**). When the phenyl group is replaced with smaller groups, the π-stacking interaction is absent, and accordingly, the stromelysin activity fell off dramatically. From the structural results, the carbonyl of the methylamide forms a hydrogen bond with alanine 169 on the stromelysin backbone as shown. The NH group on the amide does not interact with the protein, perhaps explaining the surprising potency of the tertiary amides and ester **71**. Also apparent from the X-ray results is that the amide substituent is directed toward solvent, supporting the SAR where a wide variety of substituents could be incorporated at this site with minimal loss of activity. Presumably the 1-(2-pyridyl)piperazine substituent of **66**, **69**, and **71** interacts with gelatinase A at a remote site; however this has not been confirmed by modeling.

The X-ray results of the **70**−stromelysin complex also point out why compounds from this series are selective for stromelysin. Significant enzyme differences are found for the MMPs in the unprimed side of the enzyme cleft (S1−S3), as compared to the primed side (S1′−S3′) where most of the known inhibitors bind. For instance in collagenase, a serine replaces tyrosine 155 of stromelysin, effectively eliminating the π-stacking observed for **70**. These results presumably explain why only one analogue from this series was (only slightly) active against collagenase. Furthermore, alanine 169 in stromelysin is a glutamine in collagenase, which would also not be expected to have a positive interaction with the terminal amide of **46** and analogues. In fact, the potencies obtained for several of these inhibitors against stromelysin are quite remarkable given that they do not possess substituents capable of interacting with the S1′ recognition site. Clearly, the SAR of this series combined with the X-ray results indicate that the design of a broad-based thiadiazole urea MMP inhibitor will be a major challenge.

Conclusion

The incorporation of an amino acid side chain into a thiadiazole series based on ureas **3** and **4** provided a substantial improvement in enzyme inhibition properties. In general, a substituted phenylalanine provided for superior enzyme inhibitory activity. Many analogues had K_i's of less than 0.5 μM against stromelysin, with two of the *p*-fluorophenylalanine-derived thiadiazoles, **52** and **69**, effective at 100 and 270 nM, respectively. The use of a pentafluorophenylalanine substituent provided a further gain in potency, with both **70** and **71** inhibiting stromelysin at <20 nM. The SAR observed for this series is consistent with the crystallographic results of **70** complexed to stromelysin, which, in itself, is quite unique as the inhibitor is complexed solely on the unprimed side of the enzyme cleft. Important features of this structure involve the thiadiazole urea moiety exquisitely hydrogen-bonded to the stromelysin β-sheet, with the phenylalanine group undergoing a critical π-stacking interaction with tyrosine 155. Fluoro substitution on the aryl ring improved activity considerably by enhancing the $\pi-\pi$-stacking interaction and by participating in hydrogen bonding with a structural water located near the *para*-position of the phenyl ring. The terminal amide was widely varied with minimal gain or loss of stromelysin in vitro activity. The X-ray structure is consistent with these results as the amide (or ester) substituent is directed toward solvent, with only the carbonyl group participating in hydrogen bonding to stromelysin. Most compounds within this series were selective for stromelysin. However, the incorporation of a 1-(2-pyridyl)piperazine group at the terminal amide position often enhanced gelatinase activity, with **71** having a K_i of 0.77 μM. This heterocyclic substituent, in combination with a *p*-fluoro group on the phenylalanine ring, resulted in **69**, the only analogue within this series to inhibit collagenase (13 μM). Nonetheless, a potent, broad-based inhibitor was not found within this series. The lack of broad-based

activity is consistent with the mode of binding as observed in the stromelysin complex crystal structure for **70**. Overall, the incorporation of a substituted phenylalanine side chain into a thiadiazole urea template provided a novel series of stromelysin-selective inhibitors with a potency gain of over 6000-fold attained over the screening leads. In addition, proper choice of an amide group resulted in modest gains in gelatinase potency (770 nM). The further chemical and pharmacological evaluation of this series will be reported in due course.

Experimental Section

Chemistry. Thin-layer and flash chromatography utilized E. Merck silica gel (230−400 mesh). Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Mass spectra, infrared spectra, and combustion analyses were obtained by the Physical and Analytical Chemistry Department of Pharmacia & Upjohn. ^{1}H NMR spectra were recorded at 300 MHz with a Bruker model AM-300 spectrometer.

In cases where synthetic intermediates or products were isolated by "aqueous workup (organic solvent, drying agent)", the procedure was to quench the reaction with H_2O, dilute with the indicated organic solvent, separate the organic layer, extract the aqueous layer several times with the organic solvent, dry the combined organic layers with the indicated drying agent, filter off the drying agent, and remove solvent using a rotary evaporator at reduced pressure. When "basic workup (organic solvent, aqueous basic solvent, drying agent)" is indicated, the procedure was similar to aqueous workup, except the indicated aqueous base was used instead of H_2O. When "acidic workup (organic solvent, organic solvent, drying agent)" is indicated, the procedure was to dilute the reaction mixture with the first indicated solvent, extract the organic solution several times with 10% HCl, basify the combined acidic layers with solid KOH, extract the basic solution with the second indicated organic solvent several times, dry the organic layers with the indicated drying agent, filter off the drying agent, and remove solvent using a rotary evaporator under reduced pressure. Tetrahydrofuran (THF) and ether were distilled from sodium and benzophenone. Dichloromethane was distilled from calcium hydride, and DMF was dried over 3-Å molecular sieves. All other solvents were EM Science HPLC grade, distilled in glass. Phosgene in toluene (CAUTION: phosgene is highly toxic and should be used with extreme care) was purchased from Fluka Chemie AG or Columbia. All reactions were run under nitrogen or argon.

Typical Procedure for the Preparation of Thiadiazole Amines 10. 5-(2-Phenethylamino)-1,3,4-thiadiazole-3*H*-2-thione (33). Hydrazine hydrate (1.25 mL, diluted with 1.25 mL of water) was added to a solution of phenethyl isothiocyanate (**8**, $R^1 = (CH_2)Ph$; 3.82 g, 23.4 mmol) and ethanol (25 mL) at room temperature. The solution became warm to the touch and turned slightly yellow. The solution was allowed to stir for 15 min at which time an ice bath was applied to induce crystallization. The resulting precipitate was collected by filtration, washed with ice-cold ethanol, and dried to yield 4-phenethyl-3-thiosemicarbazide (**9**, $R^1 = (CH_2)Ph$; 3.36 g, 73%) as a white solid. This material was used without further purification. Carbon disulfide (1.11 mL, 18.4 mmol) was added to a solution of 4-(2-phenethyl)-3-thiosemicarbazide (**9**, $R^1 = (CH_2)Ph$; 1.8 g, 9.2 mmol) and DMF (15 mL). The mixture was heated at 65 °C with stirring for 2 h. The solution was allowed to cool slowly and was stirred at room temperature for 48 h. The solution was then poured into water forming a precipitate, which was filtered, washed with water, and dried to yield crude product (1.95 g, 89%). This material was recrystallized from hot ethanol to give **33** (mp 146−148 °C): ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.64 (br t, 1 H), 7.18−7.33 (m, 5 H), 3.38 (m, 2 H), 3.29 (br s, 1 H), 2.83 (t, J = 7.2 Hz, 2 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 180.95, 161.18, 139.48, 129.13, 128.83, 126.69, 45.29, 34.66; MS (EI) m/z 237 (M^+), 146, 133, 91, 105. Anal. ($C_{10}H_{11}N_3S_2$).

Typical Procedure for the Preparation of Thiadiazole Ureas 13. *N*-(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-*N*′-(2-phenethyl)urea (40). A solution of 5-amino-1,3,4-thiadiazole-2-thiol (**12a**; 2.0 g, 15 mmol) and phenethyl isocyanate (**11**, $R^2 = (CH_2)_2Ph$; 2.3 g, 16 mmol) in THF (50 mL) was stirred overnight at room temperature. The THF was removed in vacuo and the residue purified by preparative reversed-phase chromatography (C18, acetone/water). Solvent was removed from the purified fractions to yield the product (2.68 g, 64%) as a white solid (mp 196−198 °C): ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.79 (br s, NH), 10.78 (br s, NH), 7.19−7.34 (m, 5 H), 6.60 (br s, NH), 3.35 (m, 2 H), 2.75 (t, J = 7.3 Hz, 2 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 183.0, 154.0, 153.3, 138.9, 128.6, 128.3, 126.1, 40.8, 35.2; MS (EI) m/z 280 (M^+), 133, 105, 91, 77, 65. Anal. ($C_{11}H_{12}N_4S_2O$).

***N*-(1,3,4-Thiadiazol-2-yl)-*N*′-(phenylmethyl)urea (15).** A solution of 2-amino-1,3,4-thiadiazole (**14**; 2.20 g, 21.7 mmol) and benzyl isocyanate (2.90 g, 21.7 mmol) in dry THF (100 mL) was stirred overnight at room temperature, during which time the product crystallized. The THF was removed in vacuo, and the residue was thoroughly triturated with ether to yield 4.77 g (94%) of the product as a white solid (mp 235−257 °C): IR (mineral oil) 3348, 2953, 2925, 2854, 1700, 1555, 1456, 1228 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.02 (br s, 1 H), 8.99 (br s, 1 H), 7.13−7.36 (m, 6 H), 4.38 (d, J = 5.9 Hz, 2 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.44, 153.64, 147.43, 139.30, 128.32, 127.10, 126.90, 43.11; MS (EI) m/z 234 (M^+), 128, 107, 106, 102, 101, 91, 77, 65, 51; HRMS (EI) calcd for $C_{10}H_{10}N_4SO$ 234.0575, found 234.0573. Anal. ($C_{10}H_{10}N_4SO$).

5-Amino-3-methyl-1,3,4-thiadiazole-3*H*-2-thione (17). 1-Methylthiosemicarbazide[24] (**16**; 235 mg, 2.23 mmol), 1,1′-thiocarbonyldiimidazole (0.660 g, 3.35 mmol), and anhydrous THF (20 mL) were combined in a dry flask under N_2. The solution was heated at reflux until complete as determined by TLC (2 h). The solvent was removed in vacuo, the residue was purified by chromatography (silica, 1% methanol in chloroform), and the final product was recrystallized from methanol/chloroform to yield **17** (199 mg, 61%) as a white solid (mp 192−193 °C): IR (ATR deposited from MeOH) 3140, 1623, 1556, 1380, 1343, 1106, 1012, 668, 625 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.26 (s, 2 H), 3.34 (s, 3 H); MS (EI) m/e 147 (M^+), 105, 72, 41. Anal. Calcd for $C_3H_5N_3S_2$: C, 24.48; H, 3.42; N, 28.54. Found: C, 24.40; H, 3.35; N, 28.78.

***N*-(4,5-Dihydro-4-methyl-5-thioxo-1,3,4-thiadiazol-2-yl)-*N*′-(2-phenethyl)urea (18).** Sodium hydride (60% dispersion in mineral oil, 34 mg, 0.85 mmol) and anhydrous THF (2.0 mL) were combined in a dry flask under N_2. The suspension was cooled to −78 °C and treated with 5-amino-3-methyl-1,3,4-thiadiazole-3*H*-2-thione (**17**; 125 mg, 0.850 mmol) in anhydrous THF (2.0 mL). After stirring for 15 min at −78 °C, a solution of phenethyl isocyanate (125 mg, 0.850 mmol) in anhydrous THF (1.0 mL) was added via syringe. The mixture was allowed to slowly warm to room temperature and was stirred overnight. The solution was carefully quenched with the addition of water and acetic acid. The THF was removed in vacuo and the residue partitioned between EtOAc (2×) and water. The EtOAc layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was recrystallized from ethanol to afford 213 mg (85%) of *N*-[4,5-dihydro-4-methyl-5-thioxo-1,3,4-thiadiazol-2-yl)-*N*′-(2-phenylethyl)-urea as a white crystalline product (mp 222−223 °C): IR (mineral oil) 3377, 1686, 1663, 1578, 1555, 1238, 1131, 700 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.94 (br s, 1 H), 7.28 (m, 5 H), 6.66 (br m, 1 H), 3.68 (s, 3 H) 3.34 (m, 2 H), 2.75 (m, 2 H); MS (EI) m/e 294 (M^+), 147, 105, 91, 71, 51, 42. Anal. ($C_{12}H_{14}N_4OS_2$).

Method A: Typical Procedure for the Formation of Thiadiazole Ester 21. *N*-[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]-L-phenylalanine Methyl Ester (51). The isocyanate of L-phenylalanine methyl ester was synthesized following the general procedure of Nowick.[25]

Thus, phosgene (7.90 mL, 15.2 mmol, 1.93 M in toluene) was added to a mixture of L-phenylalanine methyl ester hydrochloride (**19**, $R^1 = CH_2Ph$; 2.57 g, 11.9 mmol), pyridine (3.90 mL, 48.2 mmol), and CH_2Cl_2 (40 mL) at 0 °C. The mixture was stirred for 2 h at 0 °C and then diluted with EtOAc (150 mL). The organic layer was washed with cold 5% HCl (2 × 40 mL) and cold brine (50 mL). The organic layers were dried ($MgSO_4$), filtered, and concentrated to give 2.52 g of isocyanate **20** ($R^1 = CH_2Ph$) as an oil which was carried on crude.

A solution of the isocyanate (11.9 mmol) and THF (35.0 mL) was cooled to 0 °C. To this was added 5-amino-1,3,4-thiadiazole-2-thiol (**12a**; 1.60 g, 12.0 mmol). The solution was stirred at 0 °C for 1 h and at room temperature for 16 h. Aqueous workup (EtOAc, $MgSO_4$) and purification by flash chromatography (5% MeOH/CH_2Cl_2) gave 2.35 g (58%) of the urea as a yellow foam (mp 102−105 °C): $[\alpha]^{25}_D$ +56° (*c* 0.94, $CHCl_3$); IR (mineral oil) 3195, 3088, 3065, 3027, 3002, 1742, 1697, 1575, 1543, 1495, 1321, 1218, 1181, 1067, 1054, 777, 742, 702, 687 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 7.10−7.40 (m, 5 H), 6.92 (d, J = 7.7 Hz, 1 H), 4.50−4.65 (m, 1 H), 3.66 (s, 3 H), 2.90−3.15 (m, 2 H); MS (EI) *m*/z 338 (M^+), 159, 133, 120, 103, 91, 88, 65. Anal. ($C_{13}H_{14}N_4O_3S_2$).

Method B: Typical Procedure for the Formation of Thiadiazole Amide 22. α-[[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]amino]-*N*,*N*-dimethyl-(*S*)-benzenepropanamide (57). A solution of **21** ($R^1 = CH_2Ph$; 200 mg, 0.591 mmol), 40% aqueous dimethylamine (1.0 mL), and ethanol (1.0 mL) was stirred for 3 days at room temperature. The mixture was concentrated and diluted with EtOAc. The organic layer was washed with 10% HCl (2 × 15 mL) and brine (15 mL), dried ($MgSO_4$), filtered, and concentrated to provide 171 mg of crude product. Recrystallization from hot CH_2Cl_2/hexane gave 126 mg (61%) of **57** as a white solid (mp 196−197 °C): $[\alpha]^{25}_D$ +24° (*c* 0.39, DMSO); IR (mineral oil) 3332, 3167, 3129, 3063, 3027, 1693, 1622, 1576, 1547, 1495, 1422, 1405, 1320, 1283, 1231, 1065, 1054, 779, 745, 699, 637 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 13.86 (s, 1 H), 10.76 (s, 1 H), 7.10−7.35 (m, 5 H), 6.90−7.05 (m, 1 H), 4.85−5.00 (m, 1 H), 2.70−3.00 (m, 2 H), 2.88 (s, 3 H), 2.81 (s, 3 H); MS (EI) *m*/*z* 351 (M^+), 306, 159, 120, 103, 101, 91, 72. Anal. ($C_{14}H_{17}N_5O_2S_2$).

Method C: Typical Procedure for the Formation of Thiadiazole Amide 22. α-[[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]amino]-*N*-(phenylmethyl)-(*S*)-benzenepropanamide (59). A solution of **21** ($R^1 = CH_2Ph$; 200 mg, 0.591 mmol) and benzylamine (1.0 mL) was stirred for 3 days at room temperature. The mixture[31] was diluted with EtOAc and washed several times with 10% HCl (2 × 20 mL) and brine (20 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to give 214 mg of the crude amide as a solid. Recrystallization from hot CH_2Cl_2/hexane gave 150 mg (61%) of **59** as a white solid (mp 198−200 °C): $[\alpha]^{25}_D$ +13° (*c* 0.46, DMSO); IR (mineral oil) 3319, 3193, 3109, 3088, 3064, 3028, 1692, 1643, 1604, 1575, 1544, 1497, 1320, 1284, 1230, 1057, 1030, 740, 698, 645 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 13.83 (s, 1 H), 10.70 (s, 1 H), 8.65−8.80 (m, 1 H), 7.10−7.40 (m, 10 H), 6.80−6.95 (m, 1 H), 4.45−4.60 (m, 1 H), 4.15−4.40 (m, 2 H), 2.85−3.10 (m, 2 H); MS (EI) *m*/*z* 413 (M^+), 237, 163, 159, 133, 121, 120, 106, 91. Anal. ($C_{19}H_{19}N_5O_2S_2 \cdot (H_2O)_{1/8}$).

***N*-[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]-L-phenylalanine (55).** A mixture of **51** (528 mg, 1.56 mmol), MeOH (20 mL), H_2O (5.0 mL), and KOH (350 mg, 6.24 mmol) was stirred at room temperature for 16 h. The solution was concentrated (to remove the MeOH), acidified with 10% HCl, and extracted with EtOAc (3 × 20 mL). The organic layers were dried ($MgSO_4$), filtered, and concentrated to give 490 mg (97%) of acid **55**. Recrystallization from hot CH_2Cl_2/hexane provided an analytical sample as a white solid (mp 114−116 °C): $[\alpha]^{25}_D$ +15° (*c* 0.46, DMSO); IR (DRIFT) 3341, 3182, 3082, 3062, 3039, 3029, 3005, 2935, 2897, 1709, 1703, 1682, 1564, 1332, 1240 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 13.83 (s, 1 H), 13.00−13.25 (br s, 1 H), 10.81 (s, 1 H), 7.15−7.35 (m, 5 H), 6.75−6.90 (m, 1 H), 4.40−4.55 (m, 1 H), 2.95−3.20 (m, 2 H); MS (FAB) *m*/*z* 325 (MH^+), 324, 129, 71, 57, 43; HRMS (FAB) calcd for $C_{12}H_{12}N_4O_3S_2 + H_1$ 325.0429, found 325.0411.

L-*N*-(Benzyloxycarbonyl)-*O*-[(1,1-dimethylethyl)dimethylsilyloxy]serine Methyl Ester (24a). A solution of L-*N*-(benzyloxycarbonyl)serine (**23a**; 3.73 g, 14.7 mmol) in DMF (30 mL) was treated with imidazole (1.10 g, 16.2 mmol) and *tert*-butyldimethylsilyl chloride (TBDMSCl; 2.44 g, 16.2 mmol). The resultant solution was stirred for 60 h at room temperature. The DMF was removed in vacuo to give a white slurry. This material was diluted with Et_2O (100 mL) and washed with 0.5 N HCl (2×), dilute $NaHCO_3$, and brine (40 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to yield 5.33 g (99%) of **24a** as a clear, colorless oil: ^1H NMR (300 MHz, $CDCl_3$) δ 7.25−7.45 (m, 5 H), 5.58 (d, J = 3.8 Hz, 1 H), 5.13 (ABq, J_{AB} = 12.3 Hz, $\Delta\gamma$ = 6.6 Hz, 2 H), 4.35−4.45 (m, 1 H), 3.95 (d of ABq, J_d = 2.8 Hz, J_{AB} = 10.1 Hz, $\Delta\gamma$ = 67.3 Hz, 2 H), 3.74 (s, 3 H), 0.85 (s, 9 H), 0.02 (s, 3 H), 0.00 (s, 3 H).

***N*-[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]-*O*-[(1,1-dimethylethyl)dimethylsilyloxy]-L-serine Methyl Ester (27a).** A solution of the protected serine derivative **24a** (6.44 g, 17.6 mmol) in MeOH (125 mL) was treated with ammonium formate (2.22 g, 35.3 mmol) and 10% palladium on carbon (644 mg, 10 wt %). After 1 h, the mixture was filtered through Celite, washing the residual cake with MeOH (100 mL) and CH_2Cl_2 (200 mL). The filtrate was concentrated, reconstituted in CH_2Cl_2 (200 mL), filtered, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give a quantitative yield of amine **25a** as an opaque oil: ^1H NMR (300 MHz, $CDCl_3$) δ 4.45 (br s, 2 H), 3.94 (d of ABq, J_d = 3.7 Hz, J_{AB} = 10.1 Hz, Δ_γ = 23.0 Hz, 2 H), 3.75 (s, 3 H), 3.65−3.80 (m, 1 H), 0.86 (s, 9 H), 0.09 (s, 3 H), 0.04 (s, 3 H).

Following procedure A, crude amine **25a** was converted into 2.91 g (47%) of the desired material (**27a**) as an off-white, amorphous solid: $[\alpha]^{25}_D$ + 29° (*c* 1.00, DMSO); IR (mineral oil) 1697, 1668, 1578, 1542, 1490, 1349, 1322, 1259, 1212, 1108, 1075, 1058, 836, 779 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 6.89 (d, J = 8.3 Hz, 1 H), 4.35−4.50 (m, 1 H), 3.88 (d of ABq, J_d = 1.9 Hz, J_{AB} = 11.5 Hz, Δ_γ = 61.4 Hz, 2 H), 3.66 (s, 3 H), 0.82 (s, 9 H), 0.01 (s, 3 H), −0.01 (s, 3 H); MS (FAB) *m*/*z* 393 (MH^+), 335, 260, 234, 160, 134, 133, 102, 75, 73. Anal. ($C_{13}H_{24}N_4O_4SiS_2 \cdot (AcOH)_{1/3}$).

***N*-[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]-L-serine Methyl Ester (28a).** A solution of **27a** (1.03 g, 2.63 mmol) in dry THF (25 mL) was treated with tetrabutylammonium fluoride (1.0 M solution in THF, 6.30 mL, 6.30 mmol). After 1.5 h, the solution was diluted with H_2O (25 mL) and extracted into EtOAc (3 × 50 mL). The extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated to give 1.03 g of a yellow foam. The foam was purified by silica gel chromatography (2−5% MeOH/$CHCl_3$) to give 499 mg (68%) of the desired material as an amorphous, white solid. An analytical sample was crystallized from hot CH_2Cl_2/MeOH/hexane to give the desired material as white, crystalline needles (mp 180−181 °C): $[\alpha]^{25}_D$ 0° (*c* 1.01, DMSO); IR (mineral oil) 3387, 3282, 3136, 1726, 1688, 1580, 1544, 1352, 1323, 1287, 1229, 1205, 1070, 1052, 1030 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 13.80 (br s, 1 H), 10.80 (br s, 1 H), 7.03 (d, J = 8.2 Hz, 1 H), 5.25 (t, J = 5.2 Hz, 1 H), 4.25−4.35 (m, 1 H), 3.75−3.85 (m, 1 H), 3.55−3.70 (m, 1 H), 3.65 (s, 3 H); MS (EI) *m*/*z* 278 (M^+), 174, 159, 147, 133, 88, 86, 74, 60, 59, 57. Anal. ($C_7H_{10}N_4O_4S_2 \cdot (MeOH)_{1/2} \cdot (H_2O)_{1/2}$).

α-[[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]amino]-3-hydroxy-*N*-methyl-(*S*)-propanamide (29a). Following general procedure B, **28a** (353 mg, 1.20 mmol) was dissolved in a saturated solution of $MeNH_2$ in EtOH (10 mL) and stirred overnight. After workup (saturating the aqueous layer with solid NaCl), 162 mg (49%) of the title compound was recovered as a white, crystalline solid (mp 192 °C, dec): $[\alpha]^{25}_D$ +10° (*c* 0.61, DMSO); IR (mineral oil) 3325, 3198, 3110, 1706, 1644, 1553, 1490, 1415, 1327, 1261, 1227, 1060, 689, 645 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 13.80

(br s, 1 H), 10.80 (br s, 1 H), 7.90−8.05 (m, 1 H), 6.93 (d, J = 7.8 Hz, 1 H), 5.03 (t, J = 5.3 Hz, 1 H), 4.05−4.15 (m, 1 H), 3.45−3.70 (m, 2 H), 2.58 (d, J = 4.5 Hz, 3 H); MS (FAB) *m/z* 278 (MH^+), 277, 243, 242, 186, 142, 119; HRMS (FAB) calcd for $C_7H_{12}N_5O_3S_2$ 278.0381, found 278.0380. Anal. (Calcd for $C_7H_{11}N_5O_3S_2 \cdot (MeOH)_{1/2}$).

L-*N*-(Benzyloxycarbonyl)-*O*-[(1,1-dimethylethyl)dimethylsilyloxy]tyrosine Methyl Ester (24b). A solution of L-*N*-(benzyloxycarbonyl)tyrosine *O*-methyl ester (**23b**; 3.93 g, 11.9 mmol) in DMF (25 mL) was treated with imidazole (892 mg, 13.1 mmol) and TBDMSCl (1.98 g, 13.1 mmol). The resultant solution was stirred for 72 h at room temperature. The DMF was removed in vacuo to give a white slurry. This material was diluted with Et_2O (100 mL) and washed with 0.5 N HCl (2×), dilute $NaHCO_3$, and brine (50 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give a quantitative yield of the desired material as a clear, colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25−7.45 (m, 5 H), 6.90−7.00 (m, 2 H), 6.65−6.80 (m, 2 H), 5.19 (d, J = 7.7 Hz, 1 H), 5.10 (ABq, J_{AB} = 11.5 Hz, Δ_γ = 7.2 Hz, 2 H), 4.55−4.75 (m, 1 H), 3.70 (s, 3 H), 2.95−3.15 (m, 2 H), 0.97 (s, 9 H), 0.18 (s, 6 H).

***N*-[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]-*O*-[(1,1-dimethylethyl)dimethylsilyloxy]-L-tyrosine Methyl Ester (27b).** A solution of the protected tyrosine derivative **24b** (6.72 g, 15.2 mmol) in MeOH (100 mL) was treated with ammonium formate (1.92 g, 30.4 mmol) and 10% palladium on carbon (672 mg). After 3 h, the mixture was filtered through Celite, washing the residual cake with MeOH (100 mL) and CH_2Cl_2 (200 mL). The filtrate was concentrated, reconstituted in CH_2Cl_2 (200 mL), filtered, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give a quantitative yield of **25b** as a cream-colored solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.95−7.10 (m, 2 H), 6.70−6.85 (m, 2 H), 4.87 (br s, 2 H), 3.80−4.00 (m, 1 H), 3.71 (s, 3 H), 3.09 (dd, J = 13.9, 5.6 Hz, 1 H), 2.97 (dd, J = 13.9, 7.1 Hz, 1 H), 0.97 (s, 9 H), 0.18 (s, 6 H).

Following procedure A, crude amine **25b** was converted into 3.75 g (53%) of **27b** as a yellow, amorphous solid. An analytical sample was crystallized from CH_2Cl_2/hexane to give the desired material as a white crystalline solid (mp 139−142 °C): $[\alpha]^{25}_D$ +65° (*c* 1.01, DMSO); IR (mineral oil) 3382, 1726, 1689, 1575, 1542, 1512, 1329, 1285, 1272, 1256, 1215, 1051, 923, 839, 780 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.02 (d, J = 8.4 Hz, 2 H), 6.87 (d, J = 7.9 Hz, 1 H), 6.74 (d, J = 8.4 Hz, 2 H), 4.40−4.55 (m, 1 H), 3.62 (s, 3 H), 2.85−3.05 (m, 2 H), 0.91 (s, 9 H), 0.14 (s, 6 H); MS (EI) *m/z* 411, 379, 292, 250, 223, 222, 221, 159, 107, 83, 73, 59. Anal. ($C_{19}H_{28}N_4O_4S_2Si$).

***N*-[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]-L-tyrosine Methyl Ester (28b).** A solution of **27b** (2.42 g, 5.16 mmol) in dry THF (75 mL) was treated with tetrabutylammonium fluoride (1.0 M solution in THF, 12.4 mL, 12.4 mmol). After 1.5 h, the solution was concentrated to a golden oil and then diluted with H_2O (50 mL). After the aqueous layer was acidified with 6 N HCl (pH 2), the product was extracted into EtOAc (3 × 100 mL). The extracts were washed with 0.5 N HCl (100 mL) and brine (100 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated to yield 1.67 g of yellow foam. The foam was purified by silica gel chromatography (25 g, 3 → 4% MeOH/$CHCl_3$) to give 1.52 g (83%) of an amorphous, yellow solid: IR (mineral oil) 3282, 3018, 1691, 1614, 1575, 1546, 1515, 1484, 1321, 1222, 1180, 1055, 775, 729, 685 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.75 (br s, 1 H), 10.75 (br s, 1 H), 9.25 (s, 1 H), 6.92 (d, J = 8.4 Hz, 2 H), 6.80 (d, J = 7.7 Hz, 1 H), 6.66 (d, J = 8.4 Hz, 2 H), 4.40−4.50 (m, 1 H), 3.63 (s, 3 H), 2.80−3.05 (m, 2 H); MS (EI) *m/z* 216, 178, 159, 136, 133, 108, 107, 88, 77; MS (FAB) *m/z* 355 (MH^+), 354, 196, 186, 134, 88, 77; HRMS (FAB) calcd for $C_{13}H_{15}N_4O_4S_2$ 355.0535, found 355.0533.

α-[[[(4,5-Dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)amino]carbonyl]amino]-4-hydroxy-*N*-methyl-(*S*)-benzenepropanamide (29b). Following procedure B, **28b** (1.03 g, 2.91 mmol) gave 955 mg of **29b** as a white, amorphous solid. The material was crystallized from MeOH/EtOAc to give 752 mg (73%) of the desired material as a white, crystalline solid (mp 198−200 °C): $[\alpha]^{25}_D$ +72° (*c* 1.00, DMSO); IR (mineral oil) 3371, 3312, 3127, 1705, 1693, 1640, 1578, 1550, 1516, 1491, 1324, 1285, 1260, 1229, 1053 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.75 (br s, 1 H), 10.65 (br s, 1 H), 9.20 (s, 1 H), 7.95−8.10 (m, 1 H), 6.91 (d, J = 8.4 Hz, 2 H), 6.77 (d, J = 8.1 Hz, 1 H), 6.63 (d, J = 8.4 Hz, 2 H), 4.20−4.35 (m, 1 H), 2.84 (dd, J = 13.8, 5.4 Hz, 1 H), 2.69 (dd, J = 13.8, 7.5 Hz, 1 H), 2.55 (d, J = 4.5 Hz, 3 H); MS (FAB) *m/z* 354 (MH^+), 196, 195, 105. Anal. ($C_{13}H_{15}N_5O_3S_2 \cdot (H_2O)_{1/2} \cdot (C_4H_2O_2)_{1/2}$).

Biological Methods. A 33-kDa, truncated form of human stromelysin-1 (stromelysin 1−255), lacking the carboxy terminus, was cloned and expressed using methods analogous to those previously described.[32] The soluble pro-enzyme expressed in *E. coli* was pure by SDS−PAGE and amino acid analysis criteria after ammonium sulfate precipitation and isolated by anion- and cation-exchange chromatography.

Enzymatic Screening Assay. An enzymatic screening assay utilized the incubation of activated stromelysin with radiolabeled β-casein as the substrate, followed by measurement of trichloroacetic acid (TCA)-soluble casein fragments as a measure of enzyme activity. Stromelysin was activated by incubation with α-chymotrypsin. β-Casein was radiolabeled by reductive methylation using $NaCNBH_3$ and [^{14}C]formaldehyde as previously described.[33] Determinations of IC_{50} values employed test compounds at concentrations ranging from 0.1 to 2000 μM with 4−6 replicates at each concentration. The reaction was started in microtiter plates by adding 25 μL of activated stromelysin (0.01 μg) to test compounds in 25 μL of assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing labeled substrate (0.1 μCi = 0.5 μg). Assays were terminated with ice-cold TCA and centrifugation after an 18-h incubation at 37 °C. Time course experiments showed that the activity of stromelysin in this assay did not plateau until after 24 h, indicating that even slow-binding inhibitors would be detected with this method. An aliquot of the supernatant was taken for liquid scintillation counting, and data were expressed as the counts per minute released from ^{14}C-casein, after subtraction of those counts released due to nonspecific hydrolysis (wells containing buffer and substrate only). The reaction demonstrated first-order kinetics indicating that [^{14}C]-casein behaves as a single substrate and that its initial concentration was significantly less than the K_m. Actinonin was used as a reference inhibitor in the 40 assays from which the data reported here were obtained. The IC_{50} for actinonin was 0.63 ± 0.04 μM with a between assay coefficient of variation of 5.8%. This assay was the first used for this project and was subsequently replaced by the K_i assays described below. A total of 44 compounds were evaluated in both the enzymatic screening assay for determination of IC_{50} values and in the K_i assays described below for determination of K_i values. The IC_{50} and K_i values were significantly correlated (r = 0.81), with K_i values on average being slightly lower (75% of the IC_{50} value).

Continuous Fluorescence Assay. The continuous fluorescence K_i assay monitored the stromelysin-1-catalyzed hydrolysis of a fluorogenic peptide substrate. The substrate was a tryptophan-containing peptide, DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg-NH_2, internally quenched by a dinitrophenol moiety. Stromelysin was activated by heating at 50 °C for 30 min and could be stored at 4 °C for up to 1 week without loss of activity. Two milliliters of buffer (20 mM Tris-HCl, 10 mM $CaCl_2$, 10 μM $ZnCl_2$, 5 mM CHAPS, pH 7.6) was pipetted into a 3-mL quartz cuvette in a Perkin-Elmer LS-50 fluorescence spectrophotometer. Inhibitor or DMSO (20 μL) was added, followed by the addition of 10 μL of stromelysin (1.5 mg/mL). After equilibration at 25 °C for 5 min, the reaction was started by the addition of 2 μL of 0.0102 M peptide substrate. Fluorescence was measured at an excitation of 280 nM and an emission of 340 nM.

Particle Concentration Fluorescent Assay. This assay, based on that described by Mandetta,[26] was used to assess inhibitory properties of compounds against three MMPs:

collagenase, gelatinase A, and stromelysin-1. The same polypeptide substrate,[34] labeled at the amino end with fluorescein and biotin at the carboxyl end, was used with all three enzymes. Hydrolysis results in the creation of two substrate fragments: one fluorescently tagged and the other containing biotin. The assays were conducted in microtiter plates containing buffer (50 mM Tris-HCl, 150 mM NaCl, 5−22 mM $CaCl_2$, 5.55 mM CHAPS, 0−1.11 μM $ZnCl_2$, 0.02% NaN_3, pH 7.5), 100 nM substrate, and stromelysin-1 (60 nM), collagenase (80 nM), or gelatinase (10 nM) in a final volume of 100 μL. Various concentrations of inhibitor, ranging from 0.001 to 200 μM, were assayed in replicate for determination of K_i values. Enzyme and inhibitor were allowed to preincubate (15 min) at room temperature prior to the addition of substrate, followed by incubation at room temperature (stromelysin-1, 1.5 h; collagenase, 2.5 h; or gelatinase, 1.25 h). A 50-μL aliquot of the reaction was transferred to a plate containing avidin-coated polystyrene beads and then processed in a IDEXX Screen Machine 2000. Strepavidin-coated beads sequester the intact fluorescent peptide substrate, and proteolytic activity was followed by a decrease in fluorescent signal. Data were analyzed by curve fitting using an appropriate equation, and the K_i value was calculated.

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